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T Cell Division and Death Are Segregated by Mutation of TCRβ Chain Constant Domains

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Summary

We have studied the role of the T cell receptor (TCR) β chain transmembrane and cytoplasmic domains (βTM/Cyto) in T cell signaling. Upon antigen stimulation, T lymphocytes expressing a TCR with mutant βTM and Cyto domains accumulate in large numbers and are specifically defective in undergoing activation-induced cell death (AICD). The mutant TCR poorly recruits the protein adaptor Carma-1 and is subsequently impaired in activating NF-κB. This signaling defect leads to a reduced expression of Fas ligand (FasL) and to a reduction in AICD. These β chain domains are involved in discriminating cell division and apoptosis.

Introduction

Successful T cell responses depend on the expansion of a small number of T lymphocytes with receptors specific for the immunizing antigen; these antigen-specific T cells are recruited from a large pool of T cells with diverse receptor specificities. Antigen-stimulated T cells proliferate rapidly and have been observed to divide every 4 to 5 hr (Kurts et al., 1997). Unlimited T cell expansion would exclude nonproliferating T cells from the population and reduce the diversity of the T cell repertoire. To limit the total number of antigen-stimulated T cells, dividing T cells activate apoptotic signaling pathways, which result in the death of some of the expanding cells (Lenardo et al., 1999).

During initial T cell activation, stimulated T cells are resistant to apoptosis, allowing antigen-specific T cells to begin proliferating (Lenardo et al., 1999). Subsequent to several rounds of cell division, proliferating T cells become sensitive to apoptosis, resulting in activation-induced cell death (AICD) (Boehme and Lenardo, 1993; Irmler et al., 1997; Suda et al., 1996). Activated T cells express death-inducing proteins on their surface such as Fas ligand (FasL or CD95L) and Fas (CD95) (Ju et al., 1995; Sytwu et al., 1996). The role of this ligand/receptor pair has been well documented, since FasL- and Fas-deficient mice have lymphoproliferative phenotypes (Cohen and Eisenberg, 1991; Russell et al., 1993). Moreover, the majority of patients suffering from autoimmune lymphoproliferative syndrome (ALPS) have mutations in either the Fas or FasL genes, pointing to the important role of this receptor/ligand pair in regulating T cell proliferation (Rieux-Laucat et al., 1995).

The expression of FasL is controlled by TCR-generated signals, and the FasL promoter contains binding sites for the transcription factors NF-AT, NF-κB, Egr-1, Egr-2, and Egr-3, Sp-1, AP-1, ATF-2, c-Myc, and FKHRL1 (Green et al., 2003; Kavurma and Khachigian, 2003). Matsui et al. have demonstrated that after CD3 stimulation, NF-κB regulates murine FasL transcription by cooperative interactions with AP-1 and Egr (Matsui et al., 1998, 2000). This, together with other studies, suggests that NF-κB is an important factor controlling FasL induction in T cells (Hsu et al., 1999; Kasibhatla et al., 1999; Teixeiro et al., 1999).

NF-κB is induced by different receptor-generated signals that converge by phosphorylating and activating the IKK complex (Rothwarf and Karin, 1999). Recently, two signaling adaptors, Carma-1 and Bcl-10, have been shown to link the TCR and PKCθ to the activation of the IKK complex (Gaide et al., 2002; Jun and Goodnow, 2003; Pomerantz et al., 2002; Ruland et al., 2001; Wang et al., 2002). Upon TCR engagement, Carma-1 is associated with the TCR, allowing the recruitment of Bcl-10 to the membrane. This, together with PKCθ activation, leads to activation of the IKK complex and subsequent phosphorylation and degradation of the NF-κB inhibitor IκBα, resulting in NF-κB activation (Che et al., 2004; Egawa et al., 2003; Gaide et al., 2002; Jun and Goodnow, 2003; Thome, 2004; Wang et al., 2002, 2004; Zhou et al., 2004).

T cells initiate effector responses in a hierarchical manner (Itoh and Germain, 1997; Valitutti et al., 1996), but the mechanism underlying this signaling hierarchy is not completely understood. Since stimulation through the TCR is critical to both T cell proliferation and T cell apoptosis, one important issue is whether proliferation and apoptosis are mediated by distinct or related TCR signals. Relevant to this point is the finding that some antigenic peptide ligands stimulate apoptosis, but not cytokine production, in a T cell clone (Combadiere et al., 1998). Furthermore, Jurkat cells expressing a TCRβ chain with a point mutation in the transmembrane (TM)

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domain are able to secrete normal levels of IL-2 but show a defect in AICD (Rodriguez-Tarduchy et al., 1996). While this indicates that the signals for apoptosis and cytokine expression may be distinct, it does not address the relationship between proliferative and apoptotic signals originating from the TCR.

We gained some insight into this issue by generating a mutation in the TCRβ chain transmembrane and cytosolic (βTM/Cyto) domains. Transgenic mice expressing the mutant receptor displayed a lymphoproliferative phenotype. The defect was not due to an increased rate of cell division but rather to a defect in T cell apoptosis. The finding of such a mutant indicates that the TCR-mediated signals for cell division and apoptosis can be segregated.

Results

Mutation of the TCRβ Chain Transmembrane and Cytoplasmic Domains

Among the constant domains of the TCRαβ heterodimer, the βTM domain has been highly conserved during evolution. Interestingly, this domain diverged from the homologous TCRγ chain TM domain ~400 million years ago. To assess the role of βTM domain in T cell activation, we generated hybridomas expressing mutant β chain TCRs. We used cDNAs encoding the α (Vα2) and β chain (Vβ8.1/Dβ1/Jβ1.1) from the 3bbm74 TCR that is reactive to the MHC-encoded alloantigen I-A^{bm12} and Mtv-7 and Staphylococcus Enterotoxin B (SEB) superantigens (Backstrom et al., 1998; DiGiusto and Palmer, 1994). We designed two chimeric TCR β chains: the βTM/Cyto mutant construct encodes a mutant TCRβ chain, where the TCRβTM domain and the TCRβ cytoplasmic tail have been substituted with homologous sequences from Cγ1; and the βCyto mutant construct, where only the cytoplasmic tail has been replaced with Cγ1 sequences (Figure 1A). Wild-type and mutant β chain cDNAs, along with the wild-type 3bbm74 Vα2 cDNA, were transduced into the T cell hybridoma, 58 α^-/β^- (Backstrom et al., 1996). All hybridomas expressed similar amounts of surface TCR (data not shown). When tested for IL-2 or IL-3 production, βTM/Cyto mutant hybridoma cells were 10- to 100-fold more responsive to increasing doses of SEB than T cell hybridomas expressing the wild-type TCR (Figure 1B and Petersen et al. [2004]). T cell hybridomas expressing the control βCyto mutant were similar to hybrids expressing the wild-type receptor in terms of their reactivity to SEB (Figure 1B), suggesting that the mutation of the βTM domain accounts for the hyperreactive phenotype of the mutant βTM/Cyto hybridoma.

Transgenic mice expressing the 3bbm74 wild-type (Backstrom et al., 1998) and βTM/Cyto mutant receptors were generated and crossed to B6.Rag-$2^{-/-}$ mice to ensure that no endogenous TCRs would be coexpressed. Lymph node cells from mice expressing the wild-type or mutant receptors were analyzed by flow cytometry for TCR and CD4 coreceptor expression (Figure 1C). Lymph node T cells from both strains express equivalent amounts of surface TCR (Figure 1D). Immunoprecipitation assays showed that all TCR and CD3 subunits are present in the mutant TCR/CD3 complex (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/4/515/DC1/).

In both wild-type and mutant Rag-$2^{-/-}$ mice expressing the positive selecting ligand, I-A^b, a similarly high percentage of lymph node cells bear the transgenic TCR and CD4 (78% versus 86%). However, mice expressing the mutant receptor have 2-fold more TCR^+, $CD4^+8^-$ lymph node T cells compared to mice expressing the wild-type TCR ($9.4 \pm 2.5 \times 10^6$ wild-type versus $21.3 \pm 6.6 \times 10^6$ mutant T cells) (Figure 1C). In addition, the transgenic mice were crossed onto B6.C.H-2-bm12, Rag-$2^{-/-}$ mice, which express the negative selecting ligand I-A^{bm12} for this receptor. In both strains, few TCR^+, $CD4^+8^-$ lymph node T cells were found, indicating that both wild-type and mutant T cells are negatively selected (Figure 1C). Furthermore, analysis of thymocyte populations and maturation specific surface markers shows that thymic selection is intact in βTM/Cyto mutant transgenic mice (data not shown).

Exaggerated Proliferative Response in Mutant T Cells

Proliferative responses of wild-type and mutant T cells to antigens were studied in vitro. Lymph node T cells expressing the mutant receptor proliferated more vigorously in response to the MHC-encoded antigens I-A^{bm12} (Figure 2A) and I-A^d (data not shown) and the superantigens Mtv-7 (Figure 2B) and SEB (data not shown; Petersen et al., 2004) than wild-type T cells. In analogous experiments performed in the presence of saturating amounts of IL-2, similar differences were observed, implying that the enhanced proliferation of the mutant T cells is not directly related to IL-2 production (data not shown).

To ensure that this phenotype was not a particular feature of the specificity of the 3bbm74 transgenic TCR, we generated mice transgenic only for the β chain, which express TCRs comprised of either the wild-type or the mutant βTM/Cyto chain together with endogenous α chains. As with mice expressing the mutant 3bbm74 αβ receptor, transgenic mice expressing the mutant β chain contain more peripheral T cells ($27.4 \pm 5.8 \times 10^6$ wild-type versus $40.2 \pm 6.1 \times 10^6$ mutant T cells per mouse). The diversity of Vα domains expressed is also similar in the two strains (data not shown). Since both transgenic β chains encode the identical Vβ8.1DβJβ region, the corresponding T cells are responsive to SEB. Similar to mutant 3bbm74 T cells (Figures 2A and 2B), T lymphocytes expressing the mutant βTM/Cyto chain proliferate more than T cells expressing the wild-type β chain (Figure 2C). Therefore, the enhanced proliferation of the mutant T cells is a function of the mutant βTM/Cyto chain and not a peculiarity of the 3bbm74 αβ receptor.

As T cells require a period of continuous antigen exposure to commit to activation, we considered the possibility that mutant T cells might require less time in terms of antigen exposure to commit to a proliferative response (Iezzi et al., 1999). To test this idea, we analyzed proliferation of responding T cells in the presence of APCs expressing I-A^{bm12}. In these cultures, antigen presentation was terminated by adding an I-A^{bm12}-blocking mAb (M5/114) at different time points. Naive peripheral T cells from wild-type and mutant mice required at least 24 hr of continuous antigen exposure to commit to proliferation

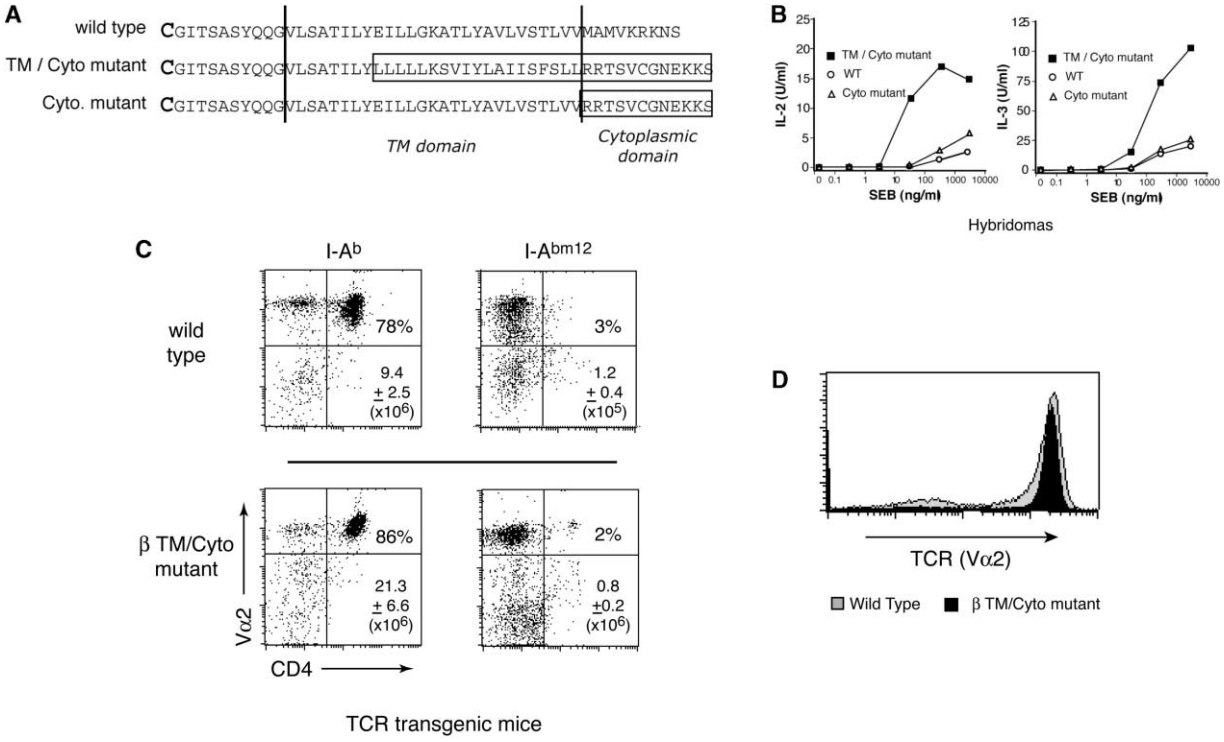

Figure 1. Hybridomas and Peripheral T Cells from Transgenic Mice Expressing βTM/Cyto Mutant TCR

(A) C-terminal amino acid sequences of wild-type and mutant β chains. Sequences are depicted beginning with the Cys, which forms the interchain disulfide bond. Amino acids within the rectangles are derived from homologous regions of the murine TCR γ chain (Cγ1).

(B) Responses of T cell hybridomas expressing the wild-type α chain along with a wild-type or a mutant β chain cultured with DAP. 3 APCs pulsed with increasing concentrations of the Staphylococcal superantigen, SEB, overnight as previously described (Backstrom et al., 1996). The data are representative of six experiments.

(C) Lymph node cells from B6 I-A^b, Rag-$2^{-/-}$, or B6 I-A^{bm12}, Rag-$2^{-/-}$ mice expressing the wild-type or β TM/Cyto mutant TCR were stained with anti-Vα2 and anti-CD4 mAbs. The number in the upper right quadrant indicates the percentage of $CD4^+$ T cells. The mean number of $CD4^+$ T cells isolated from each strain (n = 12) is indicated in each panel.

(D) Lymph node cells of B6.Rag-$2^{-/-}$ TCR-transgenic mice were harvested and stained for the transgenic α chain with mAb anti-Vα2. TCR expression on wild-type and mutant T cells is indicated by the gray and black histograms, respectively.

(Figure 2D). When exposed to antigen between 24 and 48 hr, T cells from both strains proliferated to a similar extent. However, when these cells were exposed to antigen for more than 48 hr, the mutant T cells underwent an enhanced proliferation, while the wild-type cells continued to proliferate minimally (Figure 2D). These data suggest that the increased proliferation of the mutant T cells is not due to a shortened commitment time to proliferate.

Normal Secretion of IL-2 and Expression of Early Activation Markers

Lymph node T cells were stimulated with I-A^{bm12}-expressing APCs and the kinetics of CD69 and CD25 upregulation were measured. Wild-type and mutant T cells display similar CD69 and CD25 induction kinetics (Figures 2E and 2F), indicating that many activation events are similar between wild-type and mutant T cells. Furthermore, wild-type and mutant T cells secrete comparable amounts of IL-2 (Figure 2G). It is interesting that wild-type and mutant T cells secrete similar amounts of IL-2, whereas mutant hybridoma cells clearly secrete more IL-2 than their wild-type counterparts (Figure 1B). The reason for this is unknown but may reflect the difference between ex vivo T cells and transformed hybridomas. Taken together, the enhanced proliferation of mutant T cells is neither due to enhanced IL-2 secretion nor IL-2R expression.

Accumulation of Mutant T Cells Is Not Due to Accelerated Cell Division

For assessment of proliferative responses in vivo, peripheral T cells expressing the wild-type or the mutant receptor were labeled with CFSE and adoptively transferred into T cell-deficient, B6.Rag-$2^{-/-}$ mice, which express the alloantigen I-A^{bm12} (Figure 3A). The transferred wild-type and mutant T cells did not proliferate on day 1 but did proliferate on days 2 and 3 after transfer. On each day, wild-type and mutant T cells underwent a similar number of cell divisions (Figure 3A). However, the number of recovered cells was dramatically increased in the animals receiving mutant T cells. Three days after transfer, 0.26×10^6 wild-type T cells were recovered from the spleens of B6.Rag-$2^{-/-}$, I-A^{bm12} mice compared to 4.2×10^6 mutant T cells, representing a 15-fold difference (Figure 3B). Four days after transfer, 0.46×10^6 wild-type T cells were recovered from the lymph nodes of B6.Rag-$2^{-/-}$, I-A^{bm12} mice compared to 12.2×10^6

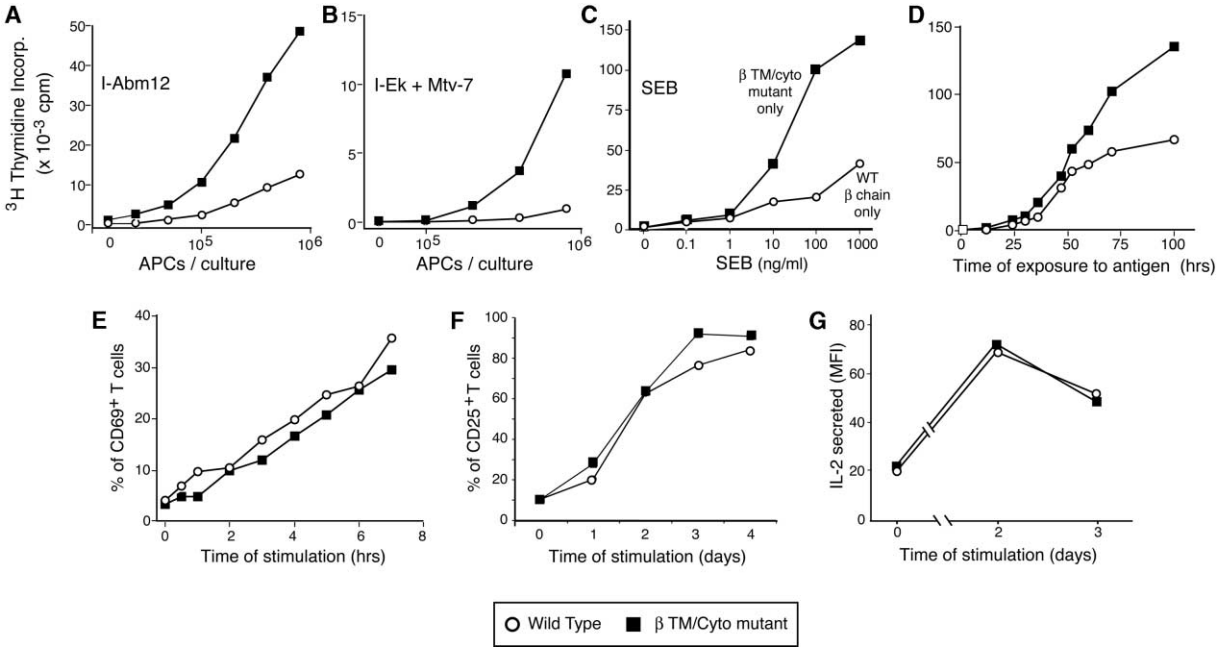

Figure 2. βTM/Cyto Mutant T Cells Show an Enhanced Proliferative Response but Normal Expression of CD69, CD25, and IL-2 Secretion

(A and B) Mixed leukocytes cultures were initiated between 2×10^5 responder lymph nodes from B6 I-A^b, Rag-$2^{-/-}$ transgenic mice expressing the wild-type (○) or βTM/Cyto mutant (■) TCRs and titrated numbers of irradiated stimulator spleen cells from (A) B6 I-A^{bm12}, Rag-$2^{-/-}$ mice or (B) Mtv-7^+ CBA/J mice.

(C) Proliferative response of lymph node T cells from B6 mice expressing the transgenic wild-type β chain (○) βTM/Cyto mutant β chain (■) along with endogenous α chains was assayed by culturing lymph node T cells from these mice with irradiated APCs and different doses of SEB as in (A).

(D) Cultures of wild-type (○) and mutant (■) T cells with APCs expressing the I-A^{bm12} antigen were initiated and at various times; the stimulation was terminated by adding a mAb (M5/114), which bound to the class II MHC molecule, I-A^{bm12}, and prevented further T cell stimulation. All cultures were maintained for 96 hr and pulsed with ^{3}H thymidine for the last 12 hr.

(E and F) T cells expressing the wild-type or βTM/Cyto mutant TCR were cultured with splenocytes bearing the I-A^{bm12} antigen. At various time points, the expression of CD69 and CD25 on the responding wild-type (○) or βTM/Cyto mutant (■) T cells was determined by flow cytometry.

(G) Wild-type (○) and βTM/Cyto mutant (■) T cells were cultured as in (E), and IL-2 secretion was determined at the indicated times by flow cytometry. There was no IL-2 response to stimulator cells from B6 (I-A^b) mice. The data in Figure 2 are representative of at least three experiments.

mutant T cells, a 25-fold difference (Figure 3C). Therefore, wild-type and mutant T cells divide at similar rates; nevertheless, significantly more mutant cells accumulate during the proliferative phase.

Mutant T Cells Have a Defect in AICD

We considered whether the striking increase in mutant T cell accumulation could be explained by a defect in AICD. The fraction of T cells undergoing apoptosis during the proliferative response to I-A^{bm12} was determined by staining the antigen-stimulated T cells with Annexin V. The data in Figure 4A show that throughout the entire proliferative response, a smaller fraction of mutant T cells undergo apoptosis compared to wild-type T cells. Since FasL is the principal death cytokine involved in the apoptosis of cycling $CD4^+$ T cells (Sytwu et al., 1996), the responding T cells were assayed for the expression of surface FasL. Mutant T cells expressed 2-fold less FasL than wild-type T cells (Figure 4B), whereas Fas receptor expression is equivalent in both cell types (Figure 4B).

Additionally, we analyzed the expression of two transcription factors, E2F-1 and p73, that have been linked to AICD (Lissy et al., 2000). As shown in Figure 4B, mutant T cells expressed approximately 2-fold less E2F-1 than wild-type cells. Since E2F-1 contributes to the induction of p73 (Irwin et al., 2000), wild-type and mutant cells showed a difference in p73 expression as well (Figure 4B). The expression of the antiapoptotic proteins Bcl-2 and Bcl-X_L (Marsden and Strasser, 2003) are similar in both types of T cells (Figure 4B).

The status of the apoptotic machinery was also evaluated. Wild-type and mutant T cells were stimulated with a variety of agents known to induce apoptosis: actinomycin D, ionomycin, ceramide, staurosporine, etoposide, and γ-irradiation. Wild-type and mutant T cells undergo apoptosis to a similar extent with all the agents tested including PMA/ionomycin, which bypasses the TCR (Figure 4C). Fas-mediated apoptosis is induced similarly in both wild-type and mutant T cells (Figure 4C). Cell death in these experiments was mediated mainly by Fas/FasL, because apoptosis was substantially inhibited in both wild-type and mutant T cells in the presence of reagents that block FasL (data not shown). These data support the idea that TCR-mediated signals are responsible for the impairment of AICD in the mutant T cells.

βTM/Cyto Mutant TCR Is Able to Discriminate between Cell Division and AICD

The availability of the I-A^{bm12} alloantigen was titrated by adding increasing amounts of 3JP, a monoclonal

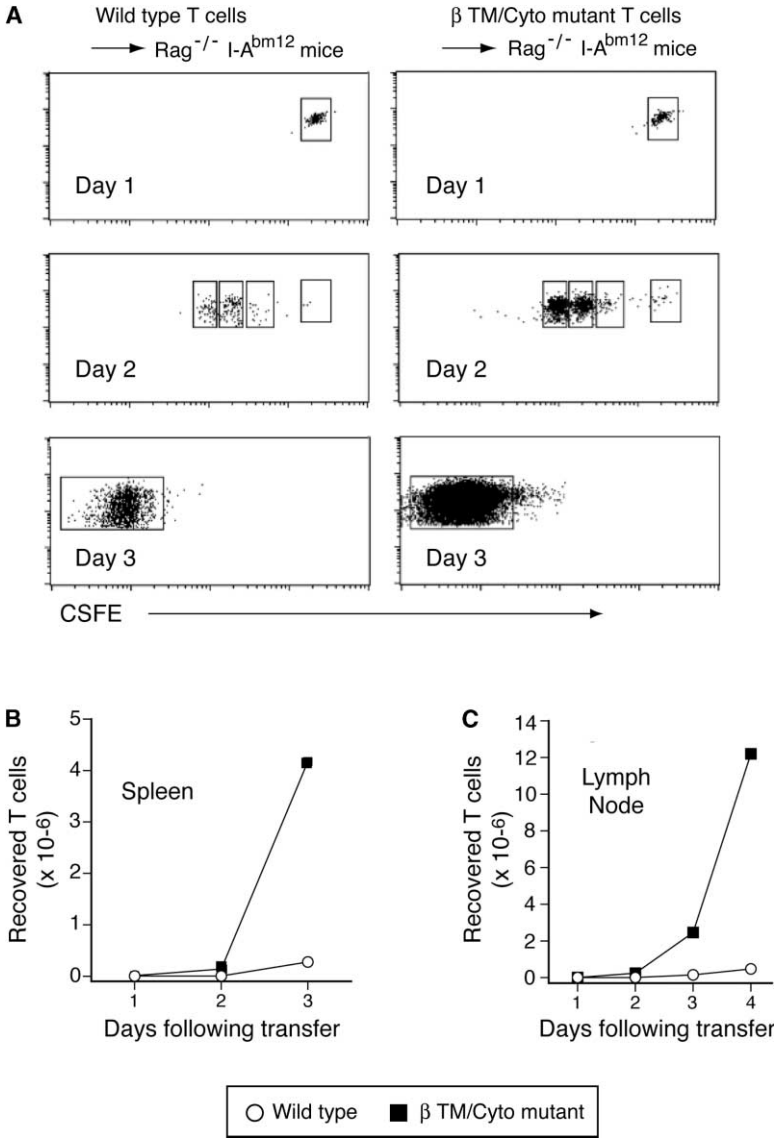

Figure 3. Proliferative βTM/Cyto Mutant Cells Accumulate In Vivo

(A) Transferred wild-type and mutant T cells divide at similar rate. Wild-type and mutant T cells were isolated, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and injected intravenously into B6 I-A^{bm12}, Rag-$2^{-/-}$ mice, which express the I-A^{bm12} antigen recognized by the transgenic T cells. On successive days after the i.v. injection of wild-type (○) or mutant (■) T cells, spleens and lymph nodes of recipient mice were harvested, and proliferation of the injected T cells was analyzed by flow cytometry.

(B and C) Cells were recovered from the (B) spleens and (C) lymph nodes of recipient mice. T cells were delineated by staining with antibodies anti-Vα2 and anti-CD4. The number of wild-type (○) and βTM/Cyto mutant (■) transgenic T cells recovered on successive days after adoptive transfer is shown. The data are representative of three experiments.

antibody that binds to I-A^{bm12} and blocks the 3bbm74 T cell response (Backstrom et al., 1998) to determine whether the defect in AICD was evident across a range of antigen concentrations. Under conditions of titrated I-A^{bm12} availability, T cell proliferation, IL-2 secretion, and AICD were examined (Figure 5). At all antigen doses, wild-type and mutant T cells show an equivalent degree of proliferation based on their CFSE profiles (Figure 5A). The division index (defined by the number of T cells entering cell division and the average number of cell divisions they undergo) is the same for wild-type and mutant T cells at all antigen doses (Figure 5B). Both types of T cells secrete similar amounts of IL-2, although mutant cells secrete more of this cytokine at some antigen doses (Figure 5C). In contrast, at all antigen doses that induce T cell death over background levels, a significantly lower fraction of mutant T cells undergo AICD than do wild-type T cells (Figure 5D). Moreover, similar results were obtained in the presence of saturating amounts of exogenous IL-2 (data not shown). These experiments rule out the possibility that mutant T cells exhibit a defect in AICD only at a single antigen dose. Furthermore, they illustrate that the signaling defect spares proliferation and IL-2 secretion and affects the induction of AICD.

Analysis of Signaling Pathways in Mutant T Cells

TCR stimulation of wild-type and mutant cells revealed no significant differences in the phosphorylation of CD3ε and ζ chains (Figure 6A). Because several reports have demonstrated that Ca^{2+} flux, ERK, JNK, and NF-κB are significant in the activation of FasL promoter (Kavurma and Khachigian, 2003), these signaling pathways were also examined. Stimulated wild-type and mutant T cells exhibit similar levels of Ca^{2+} mobilization as well as ERK and JNK activation (Figures 6B and 6C). However, mutant T cells are defective in phosphorylating and degrading the NF-κB inhibitor IκBα. TCR-induced IκBα phosphorylation is decreased in mutant cells ~3-fold, which probably leads to a delayed IκBα degradation in the mutant T cells (Figure 6D). In wild-type T cells, most of the IκBα is degraded after 15 min of stimulation (20% remaining), but in mutant T cells, IκBα begins to be degraded only after 60 min (Figure 6D). Therefore, the

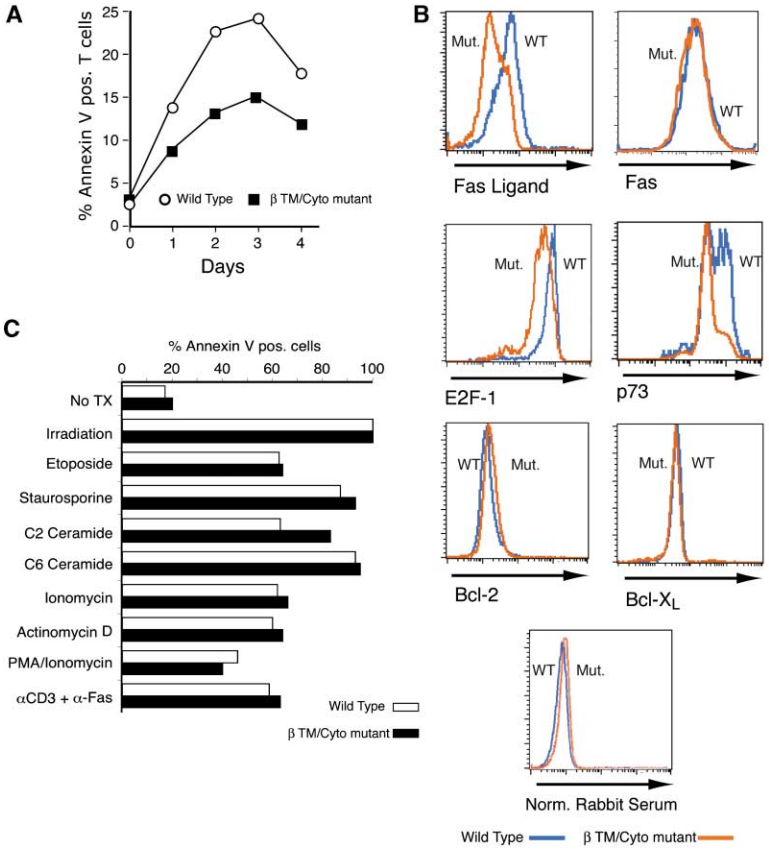

Figure 4. Defective AICD in βTM/Cyto Mutant T Cells

(A) Lymph node T cells from wild-type (○) and mutant (■) transgenic mice were cultured with I-A^{bm12}-expressing APCs for 4 days. Each day, apoptotic T cells were detected by staining with Annexin V.

(B) Lymph node T cells from wild-type and mutant transgenic mice were cultured with I-A^{bm12}-expressing APCs for 3 days, and the expression of Fas ligand (FasL), Fas, E2F-1, p73, Bcl-2, and Bcl-X_L on Vα2-expressing wild-type (blue histogram) and mutant (red histogram) T cells were determined by flow cytometry. The mean channel fluorescence (MFI) of anti-FasL-stained wild-type and mutant T cells were 50 and 23, respectively. 45% of wild-type T cells express high levels of p73 compared to only 18% of mutant T lymphocytes.

(C) Wild-type (white bars) and mutant (black bars) T cells were exposed to γ irradiation (1000 rads) or cultured with: etoposide (100 nM), staurosporine (10 nM), C2 or C6 ceramide (50 μM), ionomycin (1 μM), actinomycin D (1 nM), PMA (100–50 ng/mL) plus ionomycin (0.1 μM), and plate bound anti-CD3 (coated with 10 μg/mL) plus soluble anti-Fas MoAb Jo2 (10 μg/mL). Twenty-four hours later, apoptotic T cells were detected by Annexin V staining and flow cytometry. The data are representative of three experiments.

mutation in the TCRβ chain leads to a defect in the activation of the NF-κB signaling pathway.

Carma-1 and Bcl-10 Are Poorly Recruited to the Mutant TCR

Because Carma-1 and its binding partner Bcl-10 are proximal TCR signaling intermediates of the NF-κB signal cascade (Jun and Goodnow, 2003; Thome, 2004), we examined the ability of the mutant TCR to colocalize with Carma-1 by confocal microscopy (Figure 7A). The images show that Carma-1 efficiently cocaps with CD3 in wild-type, but not in mutant, T cells. Among stimulated T lymphocytes, which underwent CD3 capping, we observed Carma-1 cocapping in 82% of wild-type cells but in only 15% of mutant T cells ($p < 4 \times 10^{-11}$). Therefore, the mutant receptor is inefficient in recruiting Carma-1. As Carma-1 recruits Bcl-10 to the membrane upon TCR engagement (Gaide et al., 2002), the cocapping of CD3 and Bcl-10 in both types of T cells was examined. Similarly, Bcl-10 fails to colocalize with the TCR/CD3 complex in mutant T cells (Figure 7B). Cross-linking the TCR with anti-Vβ antibody generated similar results (Supplemental Figure S2). This defect may be limited to Carma-1, since other raft-associated proteins such as Lck are recruited to the mutant TCR (Figure 7C). Taken together, these data suggest that the decrease in NF-κB signaling in mutant T cells is due to the inability of the mutant receptor to recruit Carma-1.

Discussion

Upon TCR engagement, T cells become activated, acquire effector functions, and proliferate; subsequently, they either die or differentiate into memory cells. It is still not completely understood how the αβ TCR controls these distinct T cells fates. Here, we report that mutation of the transmembrane and cytoplamasmic domains of the TCR β chain causes a defect in apoptosis, but not in proliferation. This apoptotic defect is associated with a disruption in the interaction between the mutant receptor and Carma-1, which connects the surface TCR with the NF-κB signaling pathway (Jun and Goodnow, 2003; Thome, 2004). As a result, the mutant receptor is impaired in recruiting Bcl-10, in activating NF-κB, and in inducing the expression of FasL, a gene whose transcription is dependent on NF-κB induction (Kasibhatla et al., 1999; Matsui et al., 1998, 2000; Teixeiro et al., 1999; Wan and DeGregori, 2003). These data suggest that the β chain transmembrane and cytoplasmic domains are important for AICD.

Although T lymphocytes expressing the mutant receptor accumulate during a proliferative response, they don't show a decrease in the commitment time to proliferate (Figure 2). Moreover, mutant and wild-type cells are similar in terms of IL-2 secretion and IL-2R (CD25) expression (Figures 2 and 5), and they undergo cell division with equivalent kinetics (Figures 3 and 5). Finally, early activation events such as CD69 expression (Figure 2) and signaling events such as CD3ϵ/ζ chain phosphorylation, Ca^{2+} flux, and MAPK induction are unaltered in mutant T cells (Figure 6). This indicates that several signaling pathways and T cell responses remain unaffected by this TCR mutation.

There is no obvious defect in the apoptotic machinery per se, since a wide variety of pharmacological agents and anti-Fas antibodies readily induce apoptosis in mu-

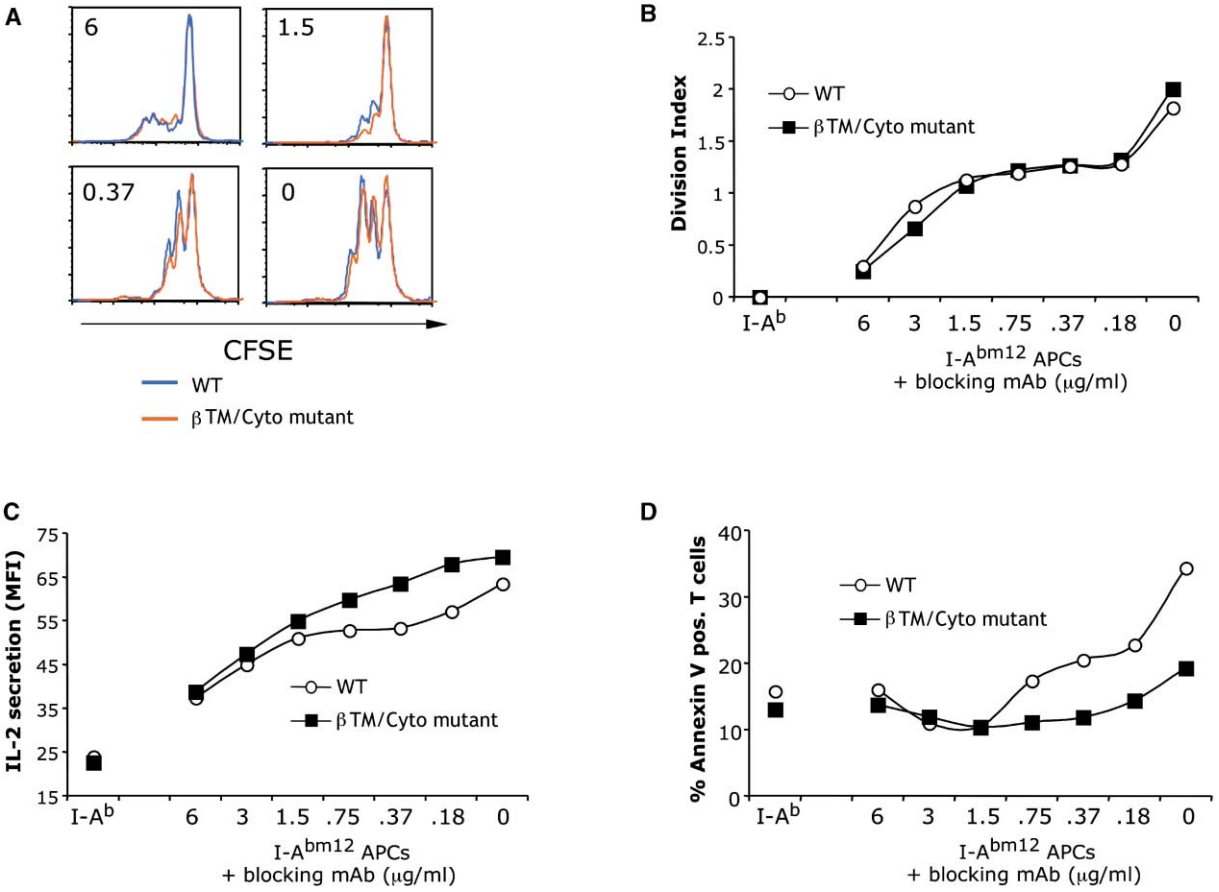

Figure 5. βTM/Cyto Mutant T Cells Are Defective in AICD Across a Broad Range of Ligand Concentrations

(A) Wild-type and βTM/Cyto mutant T cells were stained with CFSE and stimulated with I-A^{bm12} spleen APCs pulsed with the class II-blocking anti-I-A^{bm12} antibody, 3JP to provide a titration of antigen dose. Their division profile for day 3 poststimulation is shown. Wild-type profiles are shown in blue, and mutant profiles are shown in red. The number in each plot reflects the dose of blocking antibody in μg/ml. These data are representative in that the CSFE profiles of wild-type and mutant T cells are equivalent at all doses of the I-A^{bm12}-blocking mAb used.

(B) The T cell division indices of the cultures shown in (A) were determined (see Experimental Procedures) and shown as a plot of antigen dose versus division index. Wild-type is represented by (O), and βTM/Cyto mutant by (■).

(C) The same population of T cells as in (A) was tested for IL-2 secretion and is plotted as antigen dose versus MFI of secreted IL-2-positive cells. IL-2 was measure with a capture assay (see Experimental Procedures).

(D) For measurement of AICD, wild-type and mutant T cells were stimulated with irradiated I-A^{bm12} spleen cells for 3 days to induce Fas expression and restimulated with fresh I-A^{bm12} splenocytes in the presence of anti-I-A^{bm12}-blocking antibody, 3JP, at the doses indicated. After 36 hrs, AICD was assessed by Annexin V staining (day 5 postinitial stimulation). The results are plotted as antigen dose versus percentage Annexin V+ T cells. In all experiments, wild-type and mutant T cells are stimulated with B6 spleen (I-A^b) as a negative control. Data is representative of at least three experiments.

tant cells (Figure 4). The accumulation of cells seems to be due to a defect in the mutant TCR's ability to induce AICD (Figures 4 and 5). While both types of T cells express equivalent levels of Fas, mutant cells exhibit a significant impairment in the expression of FasL (Figure 4). Furthermore, apoptosis is reduced to similar levels in stimulated wild-type and mutant T cells in the presence of FasL blocking reagents (data not shown). Taken together, the accumulation of mutant T cells seems to be a consequence of the mutant receptor's inability to induce normal levels of FasL and, as a result, to properly induce FasL-mediated cell death (Figure 4). The impairment of FasL expression in βTM/Cyto mutant T cells correlates with the observed NF-κB signaling defect (Figure 6D). We and others have previously demonstrated the relevance of NF-κB in the upregulation of FasL and the presence of NF-κB binding sites in the FasL promoter (Hsu et al., 1999; Kasibhatla et al., 1999; Matsui et al., 1998, 2000; Teixeiro et al., 1999).

We have also studied the contribution of other prosurvival factors to the apoptosis-resistant phenotype. Neither Bcl-2 nor Bcl-X_L expression are altered in mutant T cells (Figure 4B). On the other hand, the expression of E2F-1 and p73, two transcription factors linked to AICD (Lissy et al., 2000), is impaired in stimulated mutant T cells (Figure 4). p73 is positively regulated by E2F-1 and antagonized by NF-κB (Irwin et al., 2000; Wan and DeGregori, 2003). Interestingly, mutant T cells display a decrease in p73 expression in spite of a decrease in NF-κB activation. The decrease in E2F-1 expression may interfere with p73 induction, regardless of the extent of NF-κB activation. Nevertheless, considering the accumulation of T cells seen in FasL-deficient mice (Cohen and Eisenberg, 1991; Russell et al., 1993), it is likely

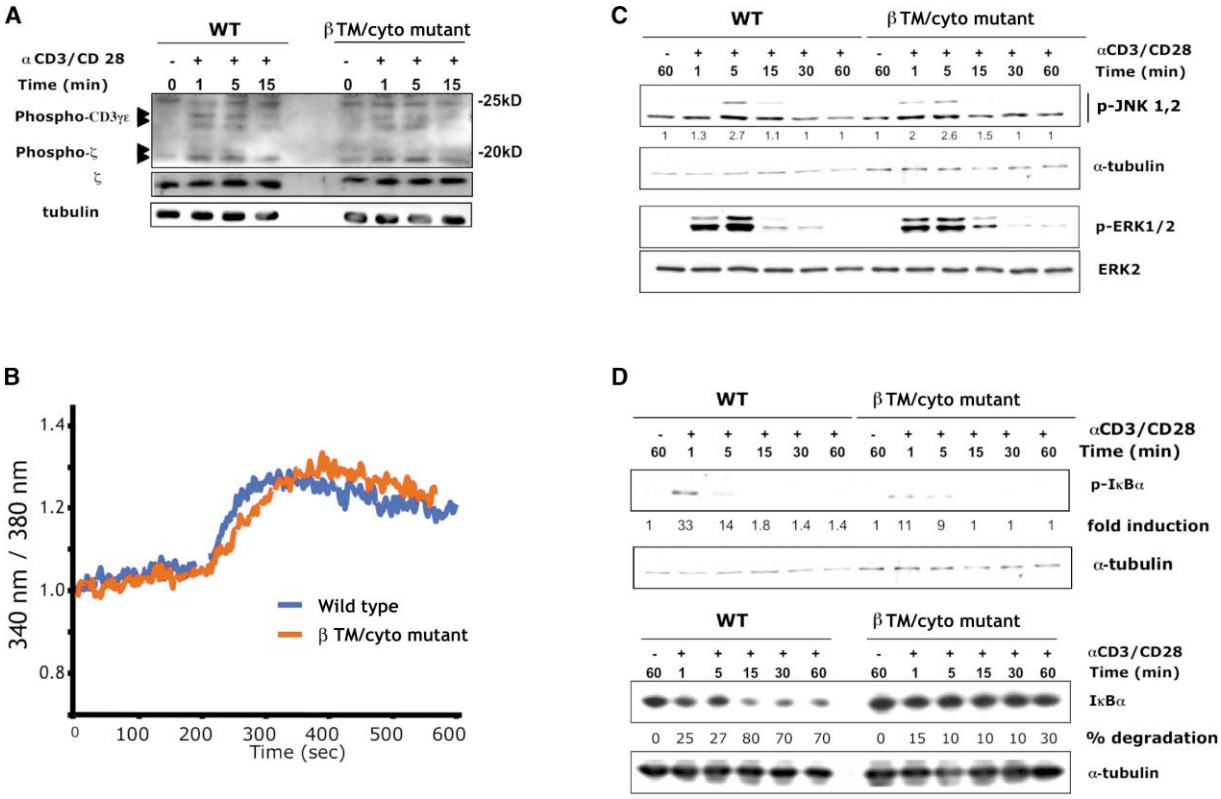

Figure 6. Mutant T Cells Show a Defect in TCR-Mediated NF-κB Signaling

(A) CD3ε and ζ chain phosphorylation was measured in stimulated T cells by Western blotting with the antiphosphotyrosine mAb 4G10. Blots were reprobed for CD3ε and ζ to confirm the identity of the phosphoproteins. ζ protein and α-tubulin are shown as loading controls.

(B) Ca^{2+} mobilization upon TCR/CD3 stimulation was measured by Fura-2 fluorescence and is shown as a ratio of the fluorescence 340 nm/380 nm.

(C) Phosphorylation of JNK and ERK was measured in stimulated T cells by Western blotting with antiphospho JNK or antiphospho ERK antibodies. Blots were reprobed for α-tubulin and ERK as loading controls. Densitometric intensity of phospho-JNK relative to nonstimulated cells is corrected for loading and shown below the panel.

(D) Phosphorylation of IκBα was analyzed as in (C) with antiphospho IκBα antibodies. The degradation of IκBα was measured by Western blot, and the densitometry of IκBα was performed as in (C).

that the decrease in FasL expression makes a major contribution to the T cell phenotype seen in βTM/Cyto mutant mice.

Carma-1 and Bcl-10 are TCR-proximal signaling adapters required for NF-κB activation (Gaide et al., 2002; Hara et al., 2003; Jun et al., 2003; Newton and Dixit, 2003; Pomerantz et al., 2002; Ruland et al., 2001; Wang et al., 2002). Upon TCR stimulation, the mutant TCR is deficient in activating the NF-κB pathway because of its poor interaction with Carma-1 (Figure 7). As a consequence, Bcl-10 is inefficiently recruited to the plasma membrane (Figure 7). Carma-1 association to lipid rafts is not affected in mutant T cells (data not shown). Furthermore, other lipid raft-associated proteins, such as Lck, colocalized normally with the mutant TCR (Figure 7). While Zap-70 inhibition does not lead to a defect in TCR/Bcl-10 association, disruption of lipid rafts with methyl-β-cyclodextran (MβCD) impairs the recruitment of Bcl-10 to the TCR (data not shown). This suggests that Carma-1 requires both the TCRβ transmembrane and cytoplasmic domains as well as lipid rafts to recruit NF-κB signaling intermediates to the membrane (Che et al., 2004; Wang et al., 2004).

Although both Carma-1 deficiency and the βTM/Cyto mutation affects IκBα degradation and NF-κB activation (Egawa et al., 2003; Gaide et al., 2002), there are striking phenotypic differences between mutant TCR- and Carma-1-deficient mice. T cells lacking Carma-1 fail to proliferate and secrete IL-2 upon TCR/CD3 stimulation, whereas T cells expressing the mutant receptor proliferate and secrete IL-2 normally (Figures 2 and 5). Carma-1-deficient T cells show a total blockade in NF-κB activation (Egawa et al., 2003; Hara et al., 2003; Pomerantz et al., 2002; Wang et al., 2002), whereas the βTM/Cyto mutant T cells only show a partial defect in NF-κB signaling. There is residual phosphorylation of IκBα at 1 and 5 min of stimulation, which apparently leads to the delay in IκBα degradation (Figure 6D). This delay correlates with the delayed cocapping kinetics of CD3 to Bcl-10 in mutant cells (data not shown). In addition, mutant T cells never fully recover their ability to colocalize this adaptor (Supplemental Figure S3). Even after 1 week in culture with antigenic APCs, mutant T cells still demonstrate a defect in Bcl-10 recruitment to the TCR and a subsequent inability to induce AICD (Figures 4 and 5; data not shown).

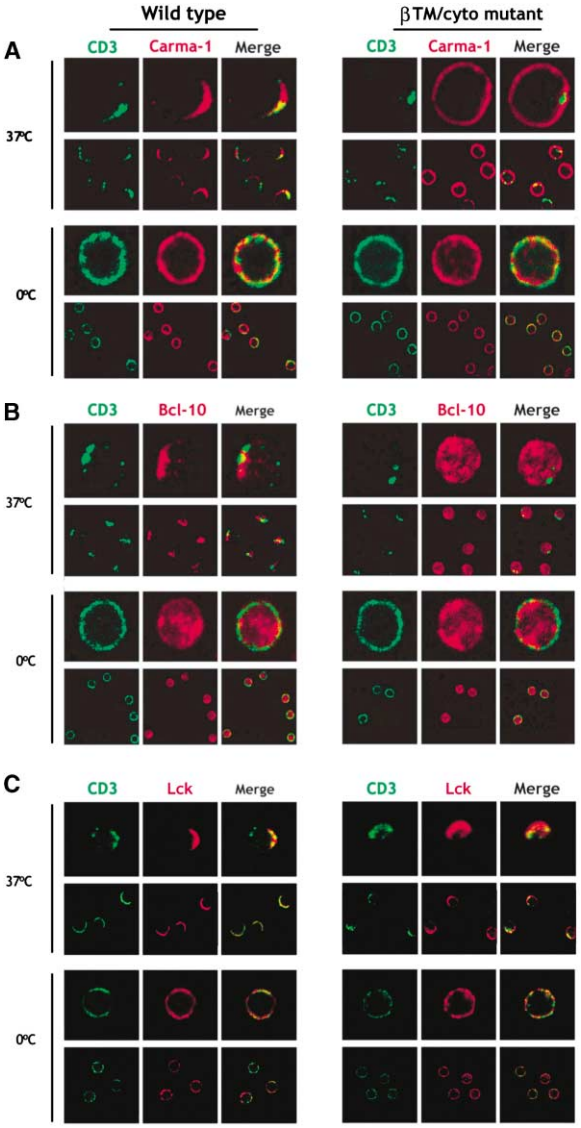

Figure 7. βTM/Cyto Mutant T Cells Are Impaired in Recruiting Carma-1 and Bcl-10 to the TCR

(A) Lymph node T cells from wild-type or mutant mice were incubated with the anti-CD3ε mAb 2C11 for 15 min. on ice followed by incubation with an Alexa488 (green)-conjugated, secondary anti-hamster antiserum for 20 min at 0°C (unstimulated) or at 37°C (stimulated). After fixation and permeabilization, the samples were stained with a polyclonal rabbit anti-Carma-1 serum followed by staining with an Alexa555-labeled, secondary goat anti-rabbit sera (red). The localization of both proteins was analyzed by confocal microscopy. Images of a representative cell and a field of cells are shown.

(B) Wild-type and mutant cells were stimulated and stained as in (A), except that red now represents staining with polyclonal rabbit antibodies for Bcl-10.

(C) Wild-type and mutant cells were stimulated for 5 min and stained as in (A) with polyclonal rabbit Lck antibodies, visualized in red.

It's unclear how the impairment of NF-κB signaling in mutant T cells specifically affects FasL expression, while other NF-κB-dependent functions such as cell division and IL-2 secretion remain unaltered. Activation of MAPKs (JNK and ERK) and Ca^{2+} flux could possibly overcome the partial NF-κB defect leading to normal cell division and IL-2 expression, but might not be sufficient for FasL induction. It is also possible that the transcriptional requirements for the FasL gene require an earlier, stronger, or more sustained activation of NF-κB. Along this line, mutation of NF-κB sites in the IL-2 promoter are less deleterious than mutations of other transcription factor binding sites for IL-2 expression (Crabtree and Clipstone, 1994; Jain et al., 1995). In the case of FasL, several reports have attributed a major role for NF-κB in FasL induction (Hsu et al., 1999; Matsui et al., 1998, 2000). Taken together, this could explain how the alteration observed in NF-κB signaling leads to a specific defect in AICD in mutant T cells.

The βTM/Cyto mutant TCR is able to discriminate cell division and IL-2 secretion from AICD (Figure 5). Several studies support the idea that different T cell functions are selectively induced based on quantitative differences in the level or duration of TCR signaling (Itoh and Germain, 1997; Valitutti et al., 1996). Therefore, we have considered whether differences in ligand affinity or blunted signals by the mutant TCR could explain the defect in AICD. This is likely not the case. First, in transgenic mice expressing a class I MHC-restricted TCR with the same βTM/Cyto mutation, no significant differences in TCR/pMHC affinity were found between the wild-type and mutant TCR (Supplemental Figure S4). Second, under conditions of titrated antigen availability, wild-type and mutant T cells show an equivalent degree of proliferation and IL-2 secretion at all antigen doses (Figure 5). In contrast, a significantly lower fraction of mutant cells underwent AICD than did wild-type T cells at all antigen doses and these differences were maintained over time (Figure 5). Taken together, these data suggest that the phenotype exhibited by mutant TCR is not a consequence of a blunted TCR, which affect all T cell responses.

Gene expression requires a coordinated activation of transcription factors. This may be particularly true for FasL, since a delay in NF-κB signaling leads to a poor induction of this death ligand. Carma-1, through its association with the TCR, plays a central role in synchronizing the induction of several signaling cascades leading to AICD.

Experimental Procedures

DNA Constructs, Viral Vectors, T Cell Hybridomas, and Cytokine Assays

cDNA constructs, viral vectors, and hybridoma lines have been described (Backstrom et al., 1996). Transduction of cDNA encoding the wild-type α chain along with cDNAs for the wild-type or mutant β chains using retroviral vectors was carried out as described (Backstrom et al., 1996). The measurement of IL-2 and IL-3 have been described (Backstrom et al., 1996, 1997).

Transgenic Mice

Transgenic mice expressing the wild-type 3bbm74 receptor have been described (Backstrom et al., 1998). cDNAs encoding the wild-type α chain and the βTM/Cyto mutant chain were cloned into the pHSE3′ expression vector and the relevant DNA fragments coinjected into C57BL/6 zygotes. Founders were repeatedly crossed to B6.Rag-$2^{-/-}$ mice to ensure that only the transgenic TCR is expressed in these animals. Two founder lines expressing the mutant receptor were established and displayed a similar phenotype. cDNAs encoding the wild-type and the βTM/Cyto mutant β chains were cloned into the pHSE3′ expression vector, and the relevant

DNA fragments separately injected into C57BL/6 zygotes to generate β chain-only mice. Animal experiments were carried out according to the cantonal and federal laws of Switzerland. Animal protocols were approved by the cantonal veterinarian of Basel, Switzerland.

Antibodies and Reagents

Monoclonal antibodies recognizing Vα2 (B20.1), CD4 (H129.19 or RM4-5), CD69 (H1.2F3), CD25 (PC6), CD5 (53-7.3), CD2 (RM2-5), CD62L (MEL-14), Fas (Jo2), Fas ligand (MFL3), I-A^{bm12} (M5/114 and 3JP), and CD28 (37.51) were purchased from PharMingen. The 2C11 mAb was kindly provided by J. Bluestone. Antisera specific for p73 (H-79) and E2F-1 (C-20) were purchased from Santa Cruz. The following antibodies were used for immunoblotting: antisera specific for ζ, CD3γ, and CD3ε (Backstrom et al., 1998); polyclonal rabbit anti-IκBα (Santa Cruz Biotechnology, Inc.); polyclonal rabbit antiphospho-IκBα (Cell Signaling, Inc.); antiphospho-ERK (Cell Signaling, Inc.); antiphospho-JNK mAb (Santa Cruz Biotechnology, Inc.); anti-ERK2 (Santa Cruz Biotechnology, Inc.); anti-phosphotyrosine mAb and 4G10 (Upstate Biotechnology); and anti-α tubulin (SIGMA) mAb. Annexin V was purchased from Pharmingen. SEB was purchased from Toxin Technologies, and CFSE (C-1157) was purchased from Molecular Probes. Alexa- and peroxidase-conjugated antibodies were purchased from Molecular Probes and Jackson Immunosearch laboratories, respectively. Actinomycin D, C6-ceramide, etoposide, ionomycin, staurosporine, and Methyl-β-cyclodextrin (MβCD) were purchased from Sigma-Aldrich Chemicals. ZAP-70 inhibitor (ZAP-70 INHIBITOR-1) was kindly provided by G. Zenke (Novartis, AG, Basel, Switzerland).

T Cell Stimulation and Flow Cytometry

Mixed leukocyte reactions between transgenic T cells and APCs were carried out as described (Backstrom et al., 1998). Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson), and the data were analyzed with CellQuest software (Becton Dickinson) or FlowJo FACS Analysis Software (Tree Star, Inc., Ashland, OR). The p73 and E2F-1 proteins were detected by intracellular staining with the Cytofix/Cytoperm intracellular staining kit (PharMingen) to fix and permeabilize cells, primary rabbit antisera specific for the p73 and E2F-1 proteins (Santa Cruz Biotechnology, Inc.), and FITC-labeled goat anti-rabbit Ig antisera (PharMingen) as a secondary staining reagent. Induction of apoptosis with anti-Fas was performed in T cells that were previously stimulated with antigen to induced Fas expression. Then, the T cells were stimulated with the anti-Fas mAb, Jo2, and additional TCR stimulation (2C11 10 μg/mL coated to plate) to initiate apoptosis as previously described (Muppidi and Siegel, 2004; Wong et al., 1997).

Calcium Mobilization

For the Ca^{2+} flux experiments, lymph node T cells were washed in Krebs buffer with 2% FCS and loaded with 5 μg/ml Fura-AM (Sigma) for 30 min at 37°C in the dark. Flux was induced by TCR crosslinking with the anti-CD3 mAb 2C11 and goat anti-hamster and measured by a Perkin Elmer fluorimeter LS50.

CFSE Labeling and Adoptive Transfer of Transgenic T Cells

Lymph node T cells (2×10^7 cells/ml) were labeled in 0.5 μM CFSE in PBS for 8 min at room temperature. Labeling was terminated by adding an equal volume of FCS and pelleting the cells by centrifugation. Cells were subsequently washed twice in medium containing 10% FCS and resuspended in PBS before intravenous (i.v.) injection.

IL-2 Secretion Assays

For determination of IL-2 secretion, T cells were stimulated with irradiated I-A^{bm12} spleen cells at a ratio of 4 APC per T cell with the Mouse IL-2 Secretion Assay Detection Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following manufacturer's instructions. Briefly, cells were washed two times in PBS + 0.5% BSA and 2 mM EDTA and stained on ice with the bispecific IL-2 catch reagent. The cells were washed twice then incubated in RPMI 1640 + 5% mouse serum at 37°C for 45 min. This was followed by two washes and incubation with the allophycorethrin-labeled α-IL-2 reagent, washed again, and analyzed by flow cytometry. Levels of IL-2 are expressed as the median fluorescent intensity (MFI).

Division Index Calculations

The division index was calculated by analyzing the CFSE profile of the populations of interest with the Proliferation Platform of FlowJo FACS Analysis Software (Tree Star, Inc., Ashland, OR). The division index represents the average number of divisions that a cell present in the starting population has undergone. The division index is (proliferation index)(%divided) where the proliferation index is the average number of divisions that those divided cells underwent, and the %divided is the percentage of the cells of the original sample that divided.

Confocal Microscopy

Lymph node T cells (0.25×10^6 cells) were washed in DMEM and incubated on ice for 15 min with anti-CD3 mAb (2C11 10 μg/mL). Cells were washed and incubated at 37°C or 0°C with conjugated Alexa488 polyclonal goat anti-hamster antibodies (Molecular Probes) in DMEM/15 mM HEPES for 20 min. Stimulation was stopped with ice-cold PBS. Cells were then spun onto TESPA (Sigma)-coated coverslips, fixed in PBS/3% paraformaldehyde for 30 min at room temperature, permeabilized for 5 min with 0.1% NP-40/PBS on ice, incubated with blocking buffer (PBS/3% BSA), and stained first, with rabbit antisera specific for Carma-1 (AL220) or Bcl-10 (Santa Cruz Bioetch., Inc.) or Lck (Cell Signaling, Inc.) and second, with conjugated Alexa555 polyclonal goat anti-rabbit antibodies (Molecular Probes) in PBS/1% BSA. After washing with PBS and 0.1× PBS, the samples were mounted in FLUORSAVE reagent (Calbiochem) and examined by confocal microscopy with a Carl ZEISS confocal microscope and the LSM510 META software (Carl Zeiss, Oberkochen, Germany).

Western Blotting

5–10 $\times 10^6$ cells for each time point in DMEM/15 mM HEPES (pH 7.5) were incubated on ice with 10 μg/ml anti-CD3 and 5 μg/ml CD28 for 15 min, followed by incubation with polyclonal goat anti-hamster antibodies at 37°C for different times of stimulation. Cell pellets were lysed in lysis buffer (20 mM HEPES [pH 7.5], 1 mM EGTA, 1% NP-40, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 40 mM β-glycerophosphate, and 2 mM sodium orthovanadate) for 30 min on ice. Cell lysates were subjected to SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked with 10% w/v skim milk in TBS-T (Tris Buffer Saline plus 0.1% Tween 20) and incubated with specific antibodies according to the manufacturer's instructions. After washing with TBS-T, the membranes were incubated with peroxidase-labeled goat anti-mouse, anti-hamster, or anti-rabbit-IgG for 1h at room temperature, and the proteins were visualized with ECL detection reagent (Amersham Corp.). Quantification of bands was performed with a densitometer (Alphainnotech, San Leandro, CA); signals were normalized to a protein (α-tubulin) serving as a loading control and compared to unstimulated samples. For the detection of ζ and CD3 chain phosphorylation, the mAb antiphosphotyrosine 4G10 was used (Upstate Biotechnology). The blot was then reprobed with anti-CD3ε- and anti-ζ-specific antibodies (Backstrom et al., 1998) to confirm the identity of the phosphoproteins and with anti-α-tubulin as a loading control.

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